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Rapid Method That Aids in Distinguishing Gram-Positive from Gram-Negative Anaerobic Bacteria

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Several species of anaerobic bacteria display variable Gram stain reactions which often make identification difficult. A simple, rapid method utilizing a 3% solution of potassium hydroxide to distinguish between gram-positive and gram-negative bacteria was tested on 213 strains of anaerobic bacteria representing 19 genera. The Gram stain reaction and KOH test results were compared with the antibiotic disk susceptibilities (vancomycin and colistin) the preliminary grouping of anaerobic bacteria. All three procedures were in agreement for the majority of strains examined. Some strains of clostridia, eubacteria, and bifidobacteria stained gram negative or gram variable; the KOH and antibiotic disk susceptibility tests correctly classified these strains as gram-positive. The KOH test incorrectly grouped some strains of *Bacteroides* sp., *Fusobacterium* sp., *Leptotrichia buccalis*, and *Veillonella parvula*, but all Gram stain results for these strains were consistent for gram-negative bacteria. The KOH test is a useful supplement to the Gram stain and antibiotic disk susceptibility testing for the initial classification of anaerobic bacteria.

The initial classification of an unknown bacterium and subsequent identification procedures are largely dependent on the results of the Gram stain. Bacteria are divided into two groups, based on their Gram stain reaction: gram positive and gram-negative. The major pitfall in the Gram staining technique is the tendency of some gram-positive bacteria to decolorize more readily than others, often resulting in these bacteria being perceived incorrectly as gram negative. Some factors, e.g., composition of the growth medium and the age of the culture (3), can influence the tendency of gram-positive bacteria to decolorize. The problem of gram-positive bacteria decolorizing is particularly evident with anaerobic bacteria; several strains characteristically stain gram negative or gram variable (6, 9).

Several modofications of the Gram stain procedure have been developed to overcome these decolorization difficulties (1-3, 7-9). In our experience, the success of these modifications with gram-positive anaerobic bacteria has been limited.

There are procedures other than staining to aid in distinguishing between gram-positive and gram-negative organisms. Cerny (2) was able to distinguish gram-negative from gram-positive facultative bacteria by assaying for aminopeptidase, a constitutive enzyme found primarily in gram-negative bacteria. Another method for the preliminary classification of bacteria is the use of a 3% solution of potassium hydroxide. Like the Gram stain reaction, the KOH test is based on differences in the chemistry of the bacterial cell wall. The cell wall of gram-negative bacteria is easily disrupted when exposed to dilute alkali solutions (4). When the cell walls are disrupted, the suspension in KOH becomes viscous due to the release of relatively unfragmented threads of deoxyribonucleic acid. Weak alkali solutions have no detectable effect on the cell wall of gram-positive bacteria. Differences in KOH solubility have been used successfully to categorize aerobic and fac- ultative bacteria, including bacteria which display variable Gram staining reactions (5).

In this investigation, the KOH test is compared with the Gram stain reaction and antibiotic disk susceptibility test for the preliminary classification (i.e., gram positive versus gram negative) of a number of genera, species, and subspecies of anaerobic bacteria.

MATERIALS AND METHODS

Bacteria. The anaerobic bacteria examined in this investigation were obtained from the stock collection of the Wadsworth Anaerobic Laboratory, Wadsworth Veterans Administration Medical Center, Los Angeles, Calif., and represent isolates obtained from a variety of sources (e.g., stools, abscesses, blood, etc.). All bacteria were identified by using standard procedures (6, 10).

Staining. Gram stains were made of cultures grown

for 48 h on supplemented (vitamin K_1 and hemin) brucella blood agar (10). Cultures of 48 h were examined in this investigation since most clinical bacteriology laboratories incubate their anaerobic culture plates for this period of time before staining. The following staining procedure was used: (i) primary staining with ammonium oxalate-crystal violet (3), 60 s; (ii) mordanting the crystal violet with an iodine solution (3), 60 s; (iii) decolorizing with 95% ethanol; and (iv) counterstaining with a 0.1% aqueous solution of basic fuchsin, 60 s.

Antimicrobial disk susceptibility. As previously described (10), we used colistin (10 μg) and vancomycin (5 μg) disks to test the antimicrobial susceptibility of all the bacteria. These two disks were placed on the surface of a supplemented brucella blood agar plate which had been inoculated with an overnight broth culture. Susceptibility to either antimicrobial agent was defined as any zone of inhibition after 48 h of anaerobic incubation at 37°C.

Potassium hydroxide test. Two drops of a 3% solution of potassium hydroxide were placed on a glass slide. We have found slides with concave wells to be very convenient for this test. A 2-mm loopful of bacterial growth, obtained from a 48-h culture on supplemented brucella blood agar, was stirred in a circular motion in the KOH solution. The loop was occasionally raised 1 to 2 cm from the surface of the slide. The KOH solution characteristically became very viscous and mucoid with gram-negative bacteria. A string of the mixture would follow the loop when it was raised (Fig. 1). The KOH test was only considered positive if stringing occurred within the first 30 s of mixing the bacteria in the KOH solution. Gram-positive bacteria suspended in the KOH solution generally displayed no reaction (absence of stringing).

Cellular lysis. The degree of cellular lysis of *Bacteroides melaninogenicus* subsp. *melaninogenicus* (false-negative KOH reaction) was compared to the lysis of *Bacteroides fragilis* (positive KOH reaction) upon exposure to 3% KOH. Equal volumes of a 6% solution of KOH and a 24-h broth culture were mixed and incubated for 15 min at room temperature. The initial absorbance at 600 nm of each cell suspension was compared with the absorbance after incubation.

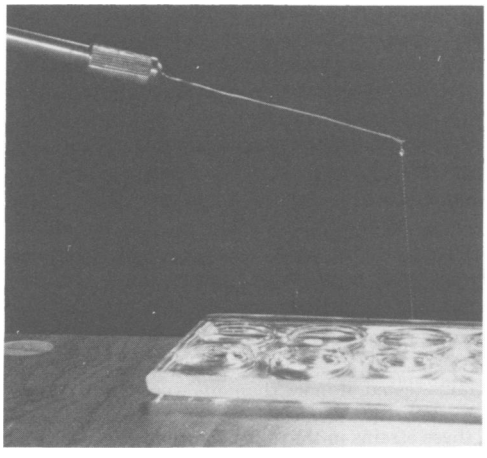

FIG. 1. *Bacteroides vulgatus mixed in a 3% solution of potassium hydroxide demonstrating a positive KOH reaction characteristic of gram-negative bacteria.*

Potassium hydroxide concentration and culture age. To examine the effect of KOH concentration and culture age on the reliability of the KOH test, we used six strains of anaerobic bacteria: *Bacteroides ovatus*, *B. fragilis*, *B. melaninogenicus* subsp. *melaninogenicus*, *B. melaninogenicus* subsp. *intermedius*, *Bacteroides ruminicola* subsp. *ruminicola*, and *Clostridium sordellii*. The KOH test was performed on these strains after 24, 48, 72, 96, and 120 h of anaerobic incubation on supplemented brucella blood agar. In addition, KOH concentrations of 2, 3, 4, 5, and 10% were tested on 48-h supplemented brucella blood agar cultures of the above strains.

RESULTS

The gram-positive and gram-negative anaerobic bacteria listed in Table 1 were correctly classified by the Gram stain reaction and the KOH test; of the 89 strains of gram-positive anaerobic bacteria studied, 72 strains stained gram positive.

Several strains of clostridia stained negative but were correctly identified as gram positive by the KOH and antibiotic disk susceptibility tests. These included four strains of *Clostridium clostridiiforme* and one strain each of *Clostridium malenominatum*, *Clostridium ramosum*, *Clostridium sphenoides*, *Clostridium sporosphaeroides*, and *Clostridium* sp. In addition to the Gram stain problem, several of these clostridial strains did not reveal spores readily upon microscopic examination.

Several strains of anaerobic bacteria gave a gram-variable reaction. These included bifidobacteria (2 strains), *Eubacterium cylindroides* (1 strain), *Eubacterium lentum* (3 strains), *Eubacterium* sp. (1 strain) and a *Lactobacillus* sp. (1 strain). The KOH reactions and antibiotic disk susceptibility patterns correctly classified these microorganisms as gram positive.

All 109 strains of gram-negative bacteria examined in this investigation were correctly classified by the Gram stain reaction (Tables 1 and 2). On the other hand, the bacteria listed in Table 2 gave KOH reactions typical of gram-positive bacteria. As can be seen from these two tables, the antibiotic disk susceptibility patterns of the gram-negative anaerobic bacteria were extremely variable. This was particularly evident with species belonging to the genus *Bacteroides*.

After 15 min of incubation at room temperature in the presence of 3% KOH, the absorbance at 600 nm of a *B. fragilis* suspension decreased approximately five times more than that of a *B.*

TABLE 1. *Species of anaerobic bacteria that gave correct reactions in the KOH test*

Organism[a]	Antibiotic disk susceptibility[b]	
	Vancomycin (5-μg disk)	Colistin (10-μg disk)
Acidaminococcus fermentans	R	S
Actinomyces meyeri	S	R
A. viscosus sp.		
Actinomyces sp.		
Anaerovibrio sp.	R	S
Arachnia propionica (2)	S	R
Bacteroides asaccharolyticus (3)	R	S
B. asaccharolyticus (2), *B. thetaiotaomicron*	S	R
B. distasonis (8)	R	R
B. fragilis (5)		
B. oralis (6)		
B. ovatus (9)		
B. putredinis (4)		
B. thetaiotaomicron (8)		
B. vulgatus (5)		
Bacteroides sp. (2)		
Bifidobacterium adolescentis (2)	S	R
B. bifidum		
B. breve		
B. infantis		
Bifidobacterium sp.	R	R
Clostridium barkeri	S	R
C. butyricum		
C. cadaveris		
C. cochlearium		
C. difficile (5)		
C. innocuum		
C. mangenotii		
C. paraputrificum		
C. perfringens		
C. sartagoformum		
C. septicum		
C. sordellii		
Eubacterium lentum (2)	S	R
E. moniliforme		
E. rectale		
Eubacterium sp. (4)		
Fusobacterium mortiferum (2)	R	S
F. naviforme (5)		
F. necrophorum (6)		
F. nucleatum (6)		
F. varium (5)		
F. naviforme (2)	R	R
Gaffkya anaerobia	S	R
Lactobacillis acidophilus	S	R
L. catenaforme (8)[c]		
L. minutus		
L. plantarum		
Lactobacillus sp.	R	R
Peptococcus asaccharolyticus	S	R
P. indolicus		
P. magnus (3)		
P. prevotii		
Peptococcus sp.		
P. asaccharolyticus	S	S
Peptostreptococcus anaerobius (5)	S	R
P. micros (3)		
P. parvulus		
P. productus		
Propionibacterium acnes (5)	S	R
P. freudenreichii[c]		
Propionibacterium sp.		
Ruminococcus albus	S	R
Selenomonas sputigena	R	S
Succinivibrio sp. (2)	R	S

[a] Numbers within parentheses indicate the number of strains tested, if more than one was tested.
[b] R, Resistant; S, sensitive.
[c] KOH test became positive after 30 s of mixing some of these strains in the KOH solution.

melaninogenicus subsp. *melaninogenicus* suspension. These results suggest that the false-negative KOH reaction seen with some of the gram-negative anaerobic bacteria (Table 2) is related to the resistance of their cell wall to KOH.

TABLE 2. *Gram-negative anaerobic bacteria incorrectly classified by the KOH test*

Organism (no. of isolates)	Antibiotic disk susceptibility[a]	
	Vancomycin (5-μg disk)	Colistin (10-μg disk)
B. asaccharolyticus (1)	R	S
B. melaninogenicus subsp. *melaninogenicus* (4)	R	R
B. melaninogenicus subsp. *intermedius* (5)	R	R
B. melaninogenicus subsp. *intermedius* (9)	R	S
B. ruminicola subsp. *ruminicola* (2)	R	R
B. ruminicola subsp. *ruminicola* (1)	R	S
F. nucleatum (1)	R	S
Leptotrichia buccalis (4)	R	S
Veillonella parvula (4)[b]	R	S

[a] R, Resistant; S, sensitive.

[b] Older cultures (>48 h) may turn positive after 30 s of mixing the bacteria in the KOH solution.

Culture age (up to 5 days) and KOH concentration (between 2 and 10%) did not have any effect on the KOH reaction of the six strains of anaerobic bacteria examined.

DISCUSSION

The preliminary classification of a bacterium as gram positive or gram negative is an essential step in both diagnostic microbiology and clinical medicine. The Gram stain is generally the first procedure performed in the identification of a bacterium and the results of this differential stain often determine the subsequent identification procedures. In addition, the type of antimicrobial treatment used in a particular infection is often based on the Gram stain reaction of the causative agent(s). A special problem is encountered with gram-positive anaerobic bacteria because many of these microorganisms readily decolorize or stain gram negative (6, 10); this is particularly evident with several species of clostridia which, in addition, often sporulate poorly in common laboratory media. These factors can occasionally result in strains of *Clostridium* sp. being incorrectly identified as *Bacteroides* sp. or *Fusobacterium* sp. For example, strains of *C. clostridiiforme* consistently stained gram negative and spores could not be detected microscopically after 48 h of growth.

The susceptibility of anaerobic bacteria to vancomycin and colistin disks is one means of differentiating gram-positive from gram-negative bacteria (10). However, the inoculated plates containing these antimicrobial disks must incubate for 24 to 72 h before the susceptibility results are known. Another procedure used to group anaerobic bacteria is gas-liquid chromatographic analysis of metabolic end products (6, 10); unfortunately, it is not available in all laboratories. As is evident from this investigation, the KOH test is a very simple and rapid means for correctly classifying gram-positive anaerobic bacteria, including those strains which readily decolorize.

All the gram-negative anaerobic bacteria examined in this investigation were correctly classified by the Gram stain. On the other hand, some of the gram-negative anaerobic bacteria gave KOH reactions typical of gram-positive bacteria (Table 2). It is interesting to note that all of the strains of *B. melaninogenicus* and *B. ruminicola* tested gave a false-negative KOH reaction, whereas most of the *B. asaccharolyticus* strains examined gave a positive KOH reaction. This false reaction could possibly be used in the rapid differentiation of *B. melaninogenicus* from *B. asaccharolyticus*. These two anaerobic bacteria closely resemble each other in preliminary classification. The false-negative reaction displayed by *B. melaninogenicus* could also be used to distinguish it from *B. oralis*. The differentiation of these two species is based primarily on pigment production; *B. melaninogenicus* produces brown to black pigment on blood agar after 4 to 7 days of incubation, whereas *B. oralis* does not (10).

The regular use of the KOH test as a supplement to Gram staining has been very effective in our laboratory in the preliminary classification of certain anaerobic bacteria. Since approximately 30% of the gram-negative bacteria gave a false-negative KOH reaction, the KOH test is useful only in conjunction with other information that may be available at the time of Gram staining (e.g., isolation medium, atmospheric requirement, colonial and microscopic morphology) or later (antibiotic disk susceptibility). The KOH test is a rapid and inexpensive supplement to the Gram stain and sometimes may be used as an alternative to antibiotic disk susceptibility testing for the initial grouping of anaerobic bacteria.

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